PHYSIOLOGY OF INSECT DIAPAUSE: THE ROLE OF THE BRAIN IN THE PRODUCTION AND TERMINATION OF PUPAL DORMANCY IN THE GIANT SILKWORM, PLATYSAMIA CECROPIA

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The phenomenon of insect diapause presents an exceptionally clear statement of one of the most important problems in biology; to wit, the nature of the factors that preside over cellular growth and differentiation. For with the onset of diapause and through the workings of internal physiological mechanisms still to be elucidated, growth suddenly comes to a standstill and the animal for months thereafter persists in a genuine state of suspended development. With the termination of diapause the rapid tempo of cellular activity returns and metamorphosis continues where it had left off. Thus whatever may be the inner mechanism for the induction and termination of diapause, it must have the capacity to turn morphogenesis off and on in a most striking way.

The study of this phenomenon has not failed to claim the attention of a large array of investigators. For example, even in 1932, Cousin was able to cite 347 papers in a review of the literature. That this extensive literature has so imperfectly advanced our knowledge of diapause is apparently due to the fact that most investigations have been carried out either on muscoid flies, which have a most imperfect and complex diapause, or on the eggs of silkworms and grasshoppers, which are too small to permit extensive manipulations of the individual animal. In the present investigation these difficulties were minimized by working on species of insects that possess a wholly characteristic pupal diapause, and, by virtue of weighing up to 8 grams per individual, are among the very largest insects in America.

Materials and Methods

Pupae of the giant silkworm, *Platysamia cecropia,* were used for the most part, approximately 1200 pupae being studied in a total of 690 experiments. These insects were reared from eggs obtained from fertile females; a lesser number of pupae were secured from dealers. In my experience, this species has never failed to enter into diapause immediately after pupation, thus giving only one brood a year. If the pupae are maintained constantly at room temperature, diapause persists for not less than five months; if they are placed immediately after pupation at a temperature of 3° to 5° C. and chilled for 1½ months or longer, adult moths emerge about 1 to 1½ months after being returned to room temperature. For this reason the stock of material was divided at the outset into two batches, one being placed and stored at 3° to 5° C. until needed ("chilled pupae"), and the other being maintained at room temperature where, as previously described, diapause persists for at least five months ("diapausing pupae").

In a number of experiments related species of saturniid pupae were used; namely, *Samia walkeri*, *Callosamia promethea*, and *Telea polyphemus*.

The most important factor facilitating the investigation was the discovery of a method of continuous anesthesia for insects during operative procedures. This method, utilizing carbon dioxide and described by Williams (1946), permitted extensive and prolonged surgical manipulations without any loss of blood or apparent damage to the pupae. Other procedures will be described as encountered in the following discussion.

Parabiotic Experiments

We have noted that diapausing pupae, after a period of exposure to low temperature, are rendered competent to develop when returned to room temperature, whereas, in contrast, pupae not subjected to chilling remain in diapause for at least five months. With these two types of animals at hand one is therefore in a position to test the fundamental nature of diapause by simply grafting one to the other so that they share the same blood. If diapause results from the *presence* of some factor inhibiting development, then such parabiotic combinations should fail to develop by virtue of the diapausing pupa distributing this inhibitory factor to the chilled individual. To the contrary, if diapause results from the *absence* of some necessary growth factor, both animals should develop, provided the chilled individual can supply double the minimal amount needed by a single animal.

In making these preparations a disc of pupal cuticle plus underlying hypodermis was cut from each pupa and the two animals placed together and held thus by the application of melted paraffin around the site. Most of the pupae were joined at the thoracic tergum (Figs. 1 and 3), but occasionally the junction was accomplished at the head or at the tip of the abdomen. Provided that the underlying heart is not injured and that no bubbles of air are trapped in either animal, such combinations are easily established and a high percentage survive.

In order to demonstrate that the operation in itself is without effects on dormancy, a series of ten diapausing pupae were successfully grafted to diapausing partners. Diapause persisted in each of these animals, adult moths being produced only after a minimum of 5½ months, the usual minimum length of time necessary for the spontaneous termination of diapause at 25° C. To the contrary, when diapausing pupae were joined to previously chilled individuals, the diapause in all viable preparations was terminated. In a series of 15 such combinations the pairs emerged as fully formed, active moths in an average of 41 days. Metamorphosis was complete both externally and internally, the only defect being a failure of the wings to expand after emergence (Fig. 2). This activation was not species- or, indeed, genus-specific, for it was possible to terminate the diapause of *Platysamia cecropia* by joining them to previously chilled pupae of *Telea polyphemus* (Figs. 3 and 4). Furthermore, sexual differences were without significance, for male pupae had the capacity to induce development of females, and vice versa.

A striking feature of all these parabiotic combinations is the fact that the animals invariably grow together so as to be connected by a pedicle, a phenomenon first noted by Crampton (1899) and later by Wigglesworth (1936) and Bodenstein (1938) in grafting procedures on insects. We shall have occasion subsequently to consider this union more fully, but in the present analysis suffice it to say that the

epithelial pedicle becomes externally chitinized and by way of its lumen permits a circulation of blood between the two animals.

In the earlier preparations the blood of the diapausing and chilled pupae in parabiosis was daily propelled to and fro by pressing accordion-like on the abdomen of each pupa alternately. This was later found to be wholly unnecessary, since development begins just as promptly without such forced mixing. It may also be noted that the completion of adult formation in the previously chilled animal occurs about 1½ days earlier than in the diapausing partner (Fig. 5). This results from a corresponding delay in the initiation of development of the diapausing pupa. At all stages in adult differentiation the chilled pupa is therefore approximately 1½ days in advance of the diapausing partner.

Thus these initial parabiotic experiments indicate some of the essential features of diapause. In general, they support the proposition that diapause results from the absence of a non-species-specific growth factor that is able to pass in parabiotic preparations from the activated to the dormant individual and evoke the initiation of adult development in the latter also.

Brain Implantation into Diapausing Pupae

If the termination of diapause is, indeed, accomplished by the action within the previously chilled pupa of a factor necessary for adult development, then it should be possible to demonstrate the organ in which this factor arises. For this purpose, various tissues and organs were removed from chilled pupae and implanted singly into diapausing individuals. When experiments of this sort were carried out, it was found that only one organ in the chilled pupa has the power to evoke development of diapausing pupae and that this organ is the brain itself. When the brain is removed from a chilled pupa and implanted into the head, thorax, or abdomen of a diapausing pupa, the latter is invariably induced to undergo adult development. Furthermore, *Platysamia cecropia* can be activated by the brains of *Samia walkeri, Callosamia promethea*, or *Telea polyphemus*—and, in fact, as far as the termination of diapause is concerned, there is a lack of species- and genus-specificity of brains among all these Lepidoptera tested. No other organ in the chilled pupa apparently possesses this power.

This effect of chilled brains is in marked contrast to that of diapausing brains,

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Explanation of Plate I

Approximately Life Size

Figure 1. Brainless, diapausing pupa of *P. cecropia* grafted to a chilled pupa of the same species.

Figure 2. Animals in Figure 1, after adult formation. The two insects have grown together and developed essentially simultaneously.

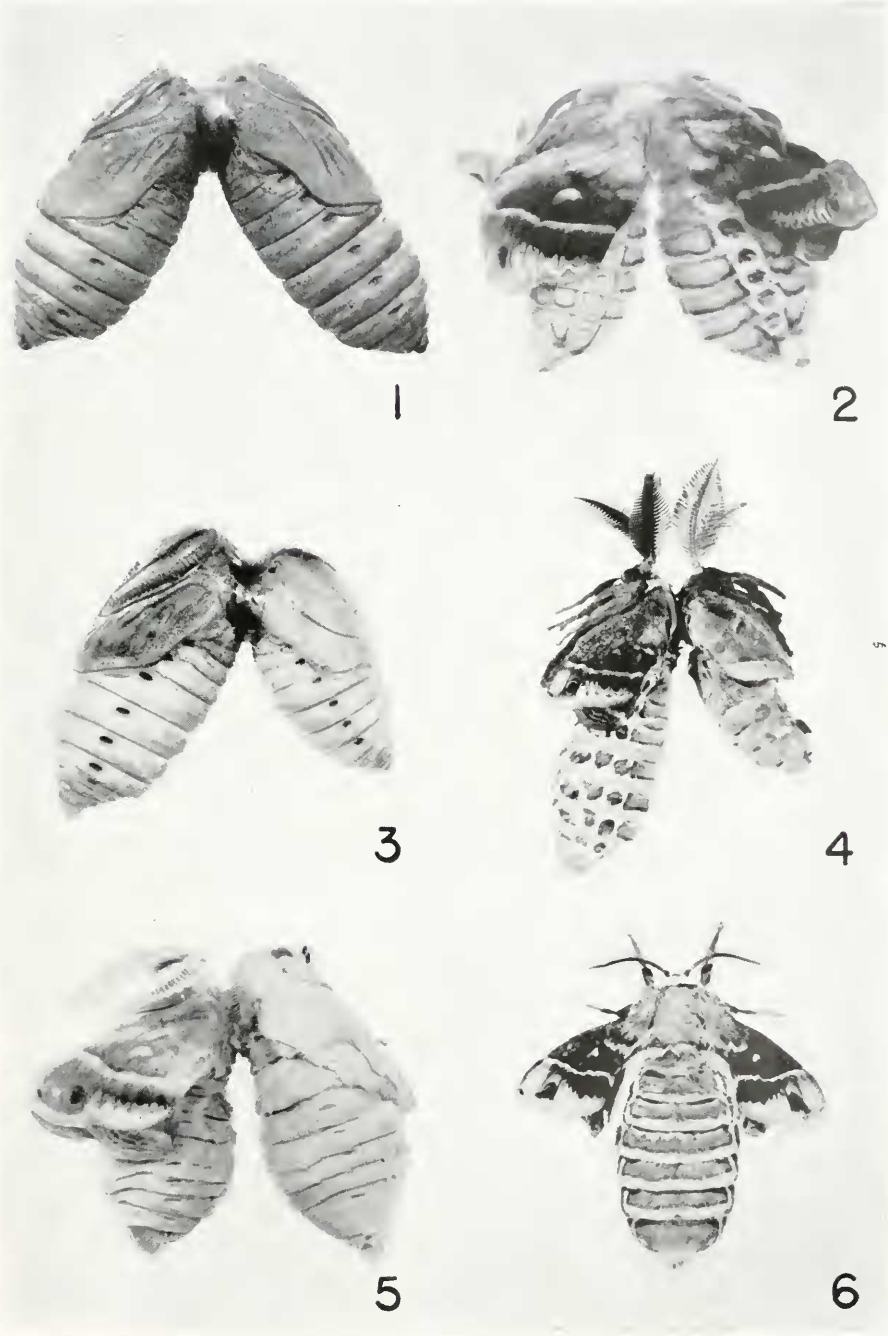
Figure 3. Brainless, diapausing pupa of *T. polyphemus* grafted to a chilled pupa of *P. cecropia*.

Figure 4. Animals in Figure 3, after adult development.

Figure 5. Parabiosis between two pupae of *P. cecropia*, removed from pupal cuticle before the completion of adult development. Development of the chilled pupa is 1½ days in advance of that of the brainless, diapausing pupa.

Figure 6. Adult Cecropia moth produced by a brainless, dipausing Cecropia pupa, whose diapause was terminated by implantation of a brain from a chilled Polyphemus pupa.

PLATE I



for the latter fail to terminate dormancy even when as many as eight are implanted into a single pupa.

The lack of species-specificity of implanted, chilled brains suggested the possibility that the effect might, conceivably, be mediated by an activation of the endogenous brain of the diapausing, "host" pupa itself. For this reason the vast majority of subsequent experiments were carried out on diapausing pupae from which the brains had been removed.

Removal of Brain

The operation devised for this purpose is easily performed, as follows. The insect, anesthetized with carbon dioxide, is placed on its back and by means of a sharp scalpel a rectangular window of pupal cuticle excised from its face. The underlying, semi-transparent hypodermis is thus exposed. With microscissors the latter is trimmed away, along with the tracheae traversing the operative field. In this procedure the frontal ganglion is frequently removed, but this has been found to be inconsequential. The brain now lies exposed and can be floated up toward the operator by pressing the pupal abdomen so that further dissection is performed in the pupal blood. With finely ground jeweler's forceps the nerves passing laterally to the site of the future adult eyes are grasped and broken. In similar fashion each brain hemisphere is carefully broken loose, in turn, from its tracheal supply, from the nerve passing posteriorly to the corpus allatum and corpus cardiacum and from the circumesophageal connective. The brain can then be lifted free and examined in insect Ringer's solution.[1] Taking care to exclude all bubbles, the defect in the pupal chitin is then capped over with a small rectangle of thin, transparent plastic (cut from a plastic cover slip), which is sealed in place with melted paraffin.

The operation was originally performed with due regard to surgical asepsis; this was later found to be of little importance since the pupae are apparently not affected by the usual contaminating organisms. The mortality from the procedure is low and one ends up with a brainless pupa possessing a transparent window at its anterior end.

The defect in the hypodermis is rapidly repaired by a deposit of blood cells, followed by an ingrowth of cells and of tracheoles along the under surface of the plastic slip. Simultaneously, an intervening, delicate, transparent, "chitinous" lamella is elaborated. These relations permit a detailed study of the behavior of the hypodermis beneath the window, and, by means of an Ultropaque, cellular activity has been followed under the oil immersion objective. It may be noted that this local process of repair occurs just as promptly in diapausing as in previously chilled pupae. Notwithstanding this fact, the process of repair is without overall effects on dormancy.

Brain Implantation into Brainless Diapausing Pupae

The removal of the brain from diapausing pupae prior to using the animals experimentally proved to be an exceptionally significant maneuver. For whereas, as

[1] This physiological solution was originally devised by Ephrussi and Beadle (1936) for studies of Drosophila, but it works equally well for the Lepidoptera used in the present experiments. I am indebted to Dietrich Bodenstein for calling my attention to its composition, which is as follows: NaCl, 7.5 gm.; KCl, 0.35 gm.; and $CaCl_2$, 0.21 gm., per liter of water.

we have previously noted, diapausing pupae kept at 25° C. begin to escape spontaneously from diapause after about five months, no such activation occurs if the brain is removed. Among approximately 400 such pupae there has not been a single case of spontaneous development. It is therefore apparent that by removing the brain the pupa is maintained in permanent diapause. Such pupae remain alive for up to a year and finally die of dessication. Yet at any time during this period the diapause can be terminated by implanting into the brainless pupa the brain of a previously chilled animal (Fig. 6). Data in regard to a series of such pupae are given in Table I.

TABLE I

Evocation of Adult Development of Brainless Diapausing Pupae by Implantation of Brains from Chilled Pupae

Species of host	Species of implanted brain	Number of experiments	Aver. time for adult emergence
P. cecropia	*P. cecropia*	16	35 days
P. cecropia	*T. polyphemus*	2	89
P. cecropia	*S. walkeri*	3	72
P. cecropia	*C. promethea*	2	63
T. polyphemus	*T. polyphemus*	2	64
T. polyphemus	*S. walkeri*	2	28

Manifestly, these experiments demonstrate that the termination of diapause requires the presence of an activated brain, in the absence of which adult development of these insects has not been observed. The conclusion is also self-evident that the termination of dormancy after diapausing pupae have been chilled results from the action of low temperature in rendering the brain able to evoke adult development. The other tissues in the diapausing pupa do not require such exposure to cold, for they are rapidly activated by implanting a brain which, alone, has been chilled.

This fact can also be readily demonstrated by removing strips of integument from diapausing pupae and implanting them into previously chilled pupae. Such diapausing tissues develop simultaneously with the host: the pupal cuticle is delaminated and a normally chitinized, adult cuticle, complete with scales and hairs, is found in the implant, in exactly the same fashion as described by Piepho (1938a and b) and Kühn and Piepho (1940) in studies of other aspects of insect metamorphosis. Thus the effect of low temperatures in facilitating escape from diapause can be explained solely on the basis of its effect on the brain.

Parabiotic Experiments on Brainless Diapausing Pupae

As soon as the brain was definitely shown to be the source of the factor terminating diapause, ten more parabiotic combinations were prepared, but this time uniting *brainless* diapausing pupae with chilled individuals. Identical results were obtained: the two pupae in each combination grew together by a chitinized, epithelial, blood-filled pedicle and after an average of 44 days emerged as normal, active, adult moths.

Brain Removal from Previously Chilled Pupae

Further information concerning the action of the brain in terminating diapause can be gained from a consideration of the behavior of chilled pupae. We have previ-

ously noted that these animals undergo no apparent development as long as they are maintained at the low temperature, but that within 1 to 1½ months after return to 25° C. the adult moth has fully formed and emerges. It is therefore worthy of note that if the brain of such chilled pupae is removed as soon as the insect is returned to the warm temperature, adult development never occurs and, in the same fashion as described for brainless diapausing pupae, dormancy persists indefinitely until the animal finally dies of dessication. Yet development can at any time be evoked by merely implanting into the head, thorax, or abdomen a brain obtained from another chilled pupa.

This phenomenon was studied more fully as follows. A series of thirty previously chilled pupae was placed at 25° C. and every few days the brains from several of these insects were removed and implanted into brainless diapausing pupae. The results may be summarized most briefly by saying that when the brain is removed within approximately the first 11 days, the brainless donors never show any development; such brains, in turn, evoke the development of brainless, diapausing pupae. In contrast, if the brain is removed from previously chilled pupae after approximately 17 days at 25° C., development continues to produce normal, brainless adults and the removed brains are without effect in terminating the dormancy of brainless, diapausing pupae.

These experiments have been repeated on a large scale, with special attention to the effect of brain removal during the critical period of 11 to 17 days. These more detailed studies were facilitated by establishing, at the outset, a transparent, plastic, facial window in each chilled pupa so that the behavior of the underlying hypodermis could be observed. It was at once apparent that the critical period, 11 to 17 days, was, in a sense, a statistical artifact, since, during this period, each individual achieves threshold activation during an extremely short interval, not exceeding a few hours. The actual critical period for each pupa is signaled by the initiation of hypodermal retraction from the overlying, facial chitin. Prior to this point, removal of the brain prevents development, and such brains evoke the development of brainless, diapausing pupae after an additional latent period of approximately three weeks. The moment hypodermal retraction is initiated, the brain can be dispensed with and such brains, when tested, are inactive.

Thus it is apparent that diapause persists even in chilled pupae until the latter have been exposed to a developmental temperature for an average of two weeks. Consequently, the activation of the pupal brain during exposure to low temperature must be conceived in terms of some physical or chemical alteration in the brain substance whereby the latter is rendered competent to produce or release its stimulating factor during subsequent exposure to a developmental temperature The brain's action is then exerted and, thereafter, metamorphosis can proceed independent of its further participation.

Role of the Corpora Allata

All the evidence so far considered reveals the brain as the organ of paramount importance in engendering and terminating diapause. Thus diapause in these species appears to result from the absence of a factor necessary for adult development, rather than from the presence of an inhibitory factor. The possibility remained, however, that the failure of the brain to exert its effect and the consequent onset

of diapause might, in turn, be due to inhibition arising elsewhere in the organism. The corpora allata were deemed the most likely source of such hypothetical inhibition and for this reason their significance in the production of diapause was studied.

As originally demonstrated by Bounhiol (1938) and subsequently confirmed by Piepho (1940; 1941), the corpora allata of Lepidoptera specifically inhibit pupation during the larval instars and thus oppose the activation of the presumptive imaginal tissues. This finding seemed so significant that comparable experiments were carried out on the caterpillars of *Platysamia cecropia* and *Telea polyphemus*. Although the removal of the corpora allata from caterpillars is a difficult procedure, it was accomplished in a sufficient number of immature (fourth instar) larvae to demonstrate that precocious pupation, indeed, results therefrom, the usual fifth instar being omitted. Furthermore, there is convincing evidence that the function of the corpora allata in inhibiting the imaginal discs disappears during the final larval instar and pupation then ensues. These findings have been considered in some detail for, although they concern pupation rather than adult differentiation, it is easy to see the importance of demonstrating whether, in potentially diapausing insects, the corpora allata once again inhibit the imaginal tissues, or the brain, and thus participate in the induction of diapause.

A series of experiments was therefore performed in which the corpora allata were removed (by a frontal approach) from diapausing pupae. Such pupae [2] invariably continued to diapause normally, and the removed corpora allata when implanted into previously chilled pupae were without effect in inhibiting adult development. As many as six corpora allata have been implanted into a single chilled pupa without retarding metamorphosis.

Similar negative results were obtained in regard to all other organs studied as a possible source of some inhibitory factor. For example, the diapausing brain itself is without inhibitory properties, for as many as six such brains have been implanted into a single chilled pupa without producing diapause. This is also true for the subesophageal ganglion, thoracic ganglion, gonads, imaginal discs, and strips of integument.

Although it cannot be denied that inhibitory factors may play a role in the production of diapause, the sum total of available evidence offers nothing to support this proposition. The brain itself remains the key to the production and termination of diapause in the species studied.

Discussion

The role of the brain in terminating diapause, demonstrated for the first time in the present investigation, can to advantage be compared with its other functions in insect metamorphosis. Thus, in the bug, Rhodnius, the brain is necessary for moulting (Wigglesworth, 1940) and in the Lepidoptera it is now well established that the brain is also required for pupation (Kopec, 1922; Caspari and Plagge, 1935;

[2] It may be noted that the allatadectomized pupae ultimately escaped from diapause after the usual minimum period of 5½ months at 25° C. The resulting moths were wholly normal in all respects and could be induced to mate and lay eggs, which, in turn, were fertile. These findings apparently deny a participation of the corpora allata in the egg production of these species, a function described for them in certain other Orders of insects (Wigglesworth, 1936; Pfeiffer, 1939).

Kühn and Piepho, 1936; Bounhiol, 1938; Piepho, 1938a; Plagge, 1938). A surprising fact is that a role of the brain in imaginal differentiation has been specifically denied by all of these investigators of lepidopteran metamorphosis. The important point is that this conclusion was, without exception, based on studies of continuous, non-diapausing development. For such insects there can be little doubt that adult formation ensues even though the brain is removed from mature caterpillars in the last instar (i.e., after the "critical period" for pupation). The existence of this striking difference between continuous and diapausing development has been pointed out previously (Williams, 1942).

In the present investigation we have seen that all the evidence supports the theory that diapause results from an interruption in the normal processes of adult development. This point of view suggests that the brain is also necessary for evoking adult development in non-diapausing pupae. In non-diapausing individuals the brain may be viewed as having achieved its full developmental function precociously prior to pupation, whereas, in potentially diapausing animals, the brain first controls pupation and then months later after pupation it controls adult formation.

The action of the brain in terminating diapause in these saturniid pupae poses an additional problem of even greater interest; namely, the nature of the factor arising in the brain which so spectacularly evokes in dormant tissues a veritable flood of cellular activity. This problem will be considered in a subsequent communication.

Summary

1. The physiological control of pupal diapause has been studied on a total of approximately 1200 pupae of the giant silkworms, *Platysamia cecropia*, *Telea polyphemus*, *Samia walkeri*, and *Callosamia promethea*.

2. The dormancy of diapausing pupae can be terminated readily by grafting them to activated (previously chilled) pupae. The two animals in each parabiotic combination grow together and some factor necessary for adult development passes from the activated to the dormant animal so that both develop simultaneously. This factor is not species- or genus-specific.

3. By implantation experiments the source of the factor terminating diapause is shown to be the brain and in this function a lack of species- and genus-specificity of brains is demonstrated.

4. In these species the well-known action of low temperatures in facilitating escape from diapause results from the effect of cold in rendering the brain competent to terminate domancy. Actual termination of dormancy is accomplished only after the previously chilled brain has been exposed to a developmental temperature for an average of two weeks. The earliest indications of adult development then become evident and the brain, thereafter, is no longer required for the completion of metamorphosis.

5. Therefore, the effect of low temperatures on the brain must consist in some physical or chemical alteration in its substance whereby the latter is rendered competent to produce or release an imaginal-differentiation factor after return to a developmental temperature.

6. No evidence was found to support the theory that diapause results from the

presence of inhibitory factors. In this regard, the functions of the corpora allata are considered in some detail.

7. It is concluded that diapause in these species results from an interruption in the normal processes of development by virtue of a failure of the brain to supply a non-species-specific factor necessary for adult differentiation. Diapause is terminated when this factor is provided.

8. The significance of the brain in the development of diapausing pupae is considered in relation to its other functions, as reported in the literature. Notwithstanding a certain amount of evidence to the contrary, it is probable that even in the absence of diapause the brain plays a vital role in adult formation.

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